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13. ABSTRACT (Maximum 200 Words)

We report a novel small protein derived from the *MUC1* gene by alternative splicing that does not contain the MUC1 tandem repeat array. This protein termed MUC1/Zs (=MUC1/ZD) retains the same N-terminal MUC1 sequences as all MUC1 protein isoforms, that comprises the signal peptide and a subsequent stretch of thirty amino acids. The MUC1/ZD C-terminal 43 amino acids are novel and result from a reading frameshift engendered by a splicing event. Expression of MUC1/ZD was demonstrated by immunohistochemistry, immunoblotting, immunoprecipitation and an ELISA assay, using MUC1/ZD specific polyclonal and monoclonal antibodies. MUC1/ZD protein was expressed in cancerous tissues and epithelial cells comprising skin tissue- its expression did not parallel the mucinous MUC1 protein. MUC1/ZD protein is expressed in tissues as an oligomeric complex composed of disulfide-linked MUC1/ZD monomers. Limited homology between the novel MUC1/ZD C-terminal 43 amino acids and the N-terminal region of CD14, an innate immunity protein, prompted investigations as to whether MUC1/ZD binds to bacteria. Using transfectants expressing the MUC1/ZD protein we demonstrated that MUC1/ZD recognized and bound to bacterial cell surfaces. Results presented here demonstrate the existence of a novel MUC1 protein isoform, MUC1/ZD, expressed in breast cancer and skin epithelial cells that participates in bacterial recognition.

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A Novel Protein Derived from the *MUC1* Gene by Alternative Splicing and Frameshifting Participates in Bacterial Binding and Recognition

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BODY-

INTRODUCTION

The classical view of mucin proteins is that they are involved in protecting the cell from ill-boding changes in the extra-cellular environment (1). These changes may be related to physical alterations such as shifts in pH, or the presence of untoward biological organisms such as harmful bacteria. Invariably, mucin proteins locate to the immediate environs exterior to the cell surface where there they may recognize and bind to harmful extracellular organisms. In this respect, mucins may be considered to be part of the innate immunity system and to act much like the carapace of a tortoise (2). Obviously there must be some sensing system external to the cell-surface, yet connected to it, that signals the cell interior about danger in the extracellular environment-the mucin proteins could well be part of this system. The cell acts accordingly by producing, amongst others, a slew of defensive molecules (3-5) including the mucin-like proteins themselves (6,7).

Genes coding for proteins with mucinous features have been grouped together as the *MUC* genes. Documentation for the existence of several *MUC* genes, designated *MUC1* to *MUC19*, has been presented to date. Just arguments exist for the assembly of these diverse genes as coding for a family of proteins. The prime case in favor cites the fact that all of the mucin proteins deriving from the *MUC* genes comprise serine and threonine rich domains that are heavily O-linked glycosylated. These regions often appear as head-to-tail tandem repeats, each repeat comprising several to tens, or even hundreds, of amino acids. The heavily O-linked glycosylated domains, render a very high molecular mass to the mature mucin protein.

Mucinous proteins derived from the *MUC* genes can be divided into those that are secreted from the cell (secreted mucins) and the mucins that comprise a transmembrane domain that anchors them to the cell membrane (transmembrane mucins). The secreted mucins include the mucin genes *MUC2, MUC5AC, MUC5B* and *MUC6* that form a cluster on chromosome band 11p15.5 and code for gel-forming mucins (8), as well as the *MUC7* and *MUC9* genes which encode relatively small mucins expressed in salivary glands and Fallopian tubes, respectively (9,10). The *MUC1, MUC3, MUC4, MUC12* and *MUC13* genes code for transmembrane mucins (11-17). The *MUC3* and *MUC12* genes are clustered on chromosome band 7q22 and code for transmembrane mucins expressed primarily in the small and large intestine, respectively. Some *MUC* genes may code for both protein varieties, secreted and transmembrane, by employing either post-translational mechanisms, such as proteolytic cleavage (18-20) that may release a membrane-tethered mucin, or by alternative splicing mechanisms.

The archetype membrane-tethered mucin, and the one to be first characterized and sequenced, is derived from the *MUC1* gene (11,21-23). The major protein derived from the *MUC1* gene is a transmembrane protein that contains the mucinous serine-threonine-rich tandem repeat region in its extracellular domain (Figure1A, MUC1/REP). This protein also comprises a 72 amino acid tail that can be tyrosine phosphorylated and subsequently interacts with second messenger proteins (24-28). This conveys a signal to the nucleus thereby altering the repertoire of genes expressed. It may be that the tandem-repeat-array-containing membrane-bound MUC1 is acting as an extracellular sensor that following appropriate stimulation, such as for example interaction with bacteria, alters nuclear gene expression. Indeed, application of bacteria to MUC1-expressing epithelial cells rapidly induces considerable tyrosine phosphorylation of the MUC1 cytoplasmic tail.[1] It thus appears as if the transmembrane MUC1/REP protein has combined two important facets within the one protein- that of a protective extracellular mucinous protein together with a protein incorporating classical signaling features.

Challenging the classical definition of a mucin gene was the discovery of MUC1 mRNAs, that although being transcribed from a mucin gene, are devoid of a tandem-repeat-array (29-32). Alternative splicing generates these MUC1 derived mRNAs that no longer code for tandem-repeat-array-containing mucinous proteins. For the *MUC1* gene several different groups have independently confirmed this finding (31,32). The prime

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[1] M. A. McGuckin, personal communication.
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example is the tandem-repeat-array-deleted MUC1/Y isoform, which is expressed both as mRNA and protein (29,30). MUC1/Y protein is similar to MUC1/REP in that it also contains the transmembrane and cytoplasmic domains, yet it lacks the central tandem repeats and their flanking sequences (Figure 1A).

We report here an additional alternatively spliced MUC1 mRNA that also deletes the central tandem-repeat-array. Following the discovery of the tandem-repeat-array-lacking MUC1/Y isoform this finding is not totally unexpected. What is surprising is the fact that downstream to the splice acceptor site, the usual MUC1 reading frame has undergone a +1 reading frameshift. This creates the potential for a novel MUC1 protein, designated here as MUC1/ZD, that comprises a completely new C-terminal protein sequence different to all other MUC1 proteins.

Neither the existence of this MUC1 isoform as an expressed protein nor its function has been previously documented. We show here that the putative MUC1/ZD protein is unequivocally expressed. Moreover, it is shown that MUC1/ZD plays a key role in recognition and binding of bacteria. These findings resonate well with the view that proteins derived from *MUC* genes may be involved in protecting the cell from harmful extracellular organisms.

BODY-

EXPERIMENTAL PROCEDURES

RT-PCR identifying the MUC1/ZD isoform. Poly(A)-rich RNA extracted from breast cancer cells was reverse transcribed (SuperscriptII, GibcoBRL) using the reverse antisense oligonucleotide primer 5'TGGCACATCACTCACG CTGACGTCTGAGATC3' that is complimentary to nucleotides 1243-1272 in the MUC1/REP sequence (for nucleotide numbering see Figure 3B in ref. 29) and 10% of the reverse transcription reaction was subjected to PCR amplification (AmpliTaq Gold, Applied BioSystems) with the same reverse antisense primer and the forward sense oligonucleotide primer 5'GAATCTGTTCTGCCCCCTCCCCAC3' corresponding to MUC1/REP nucleotides 18-42. Thirty-five PCR cycles of 92°C for 60 seconds, 60°C for 60 seconds and 72°C for 60 seconds were employed. MUC1-specific RT-PCR products were subcloned into M13 and sequencing was accomplished using the dideoxynucleotide chain termination method. Priming was with either the M13 universal primers or synthetic oligonucleotides prepared according to known sequences. Sequencing identified the novel MUC1/ZD sequence reported here (Figure 1), as well as the previously documented MUC1/Y sequence (29).

Cloning of MUC1/ZD in eucaryotic expression vector. The MUC1/ZD DNA (Figure 1) was subcloned into the ppolyII vector (33) for plasmid propagation in bacteria and for making stable eucaryotic cell transfectants, into the eucaryotic expression vector pCL642 (34) that utilizes the housekeeping gene hydroxymethylglutaryl-coenzyme-A-reductase promoter, generating the plasmid pCL-MUC1/ZD

Generation of stable 3T3 transfectants expressing MUC1 proteins. MUC1/ZD transfectants- 3T3 cells were seeded in 9cm culture Petri dishes and cotransfected with the eucaryotic expression plasmid pCL-MUC1/ZD and pSV2neo (coding for neomycin resistance) using the transfection reagent DOTAP (Roche Applied Science). Stable transfectants were selected by growing the cells with the selecting neomycin antibiotic at a final concentration of 1mg/ml. Transfectants expressing the MUC1/ZD protein were identified by fluorescence cell sorting analyses (FACS) using the MUC1/ZD specific monoclonal antibody ZUM12D8 (see below). **MUC1/REP** (see Fig. 1) **transfectants-** 3T3 cells were seeded in 9cm culture Petri dishes and cotransfected with the eucaryotic expression plasmid pCL-MUC1/REP and pSV2neo (coding for neomycin resistance) using the calcium-phosphate transfection method. Stable transfectants were selected by growing the cells with the selecting neomycin antibiotic at a final concentration of 1mg/ml. Transfectants expressing the MUC1/REP protein were identified by fluorescence cell sorting analyses (FACS) using the MUC1/REP specific monoclonal antibody H23 which recognizes an epitope contained within the twenty amino acid tandem repeat (see below).

SDS-Polyacrylamide Protein Gel Electrophoresis (SDS-PAGE). SDS-polyacrylamide gel for protein separation was performed as previously described (36). Prior to their loading, protein samples were mixed with SDS-sample buffer either in the presence or absence of reducing agents (final concentrations of 100mM of DTT and/or 150mM β-mercaptoethanol) and boiled for 5 minutes. Protein separation on the gel was performed over night at 40V in SDS- running buffer.

Two dimensional SDS-PAGE diagonal gel electrophoresis. Samples analyzed by diagonal SDS-PAGE were resolved in two dimensions following a modification of a previously described procedure (37). The first dimension was performed under non-reducing conditions- disulfide linked proteins will thus migrate according to their combined molecular masses. For the second dimension desired lanes were excised from the first gel, incubated with SDS loading buffer with or without 100mM DTT and reloaded horizontally on a second gel. After reduction of their disulfide S-S bonds, proteins will migrate according to their monomeric molecular mass.

Transfer of proteins to nitrocellulose membranes and immunoblotting analysis.
Proteins, separated on SDS PAGE, were electro-transferred at 0.5Amp for 2 hr to a nitrocellulose filter paper in transfer buffer according to Gershoni (38). The blots were blocked in PBS containing 5% skimmed milk followed by incubation with primary antibody overnight at 4°C. The filters were washed in PBS containing

0.1% Brij and then incubated with a secondary anti-mouse antibody conjugated to horseradish peroxidase followed by washing with PBS/Brij and incubation with enhanced chemiluminescent substrate.

Immunoprecipitation. Immobilized antibody complexes were prepared by incubating 10µl (bed volume) of protein A (PAA) or Protein G agarose (Repligen) with 5-10 µg of antibody. PBS was then added to a final volume of 0.5 ml and the beads incubated for 1.5 hr at 4°C with shaking. Preclearing of lysate proteins was performed by incubating lysates (at a protein concentration of approximately 1mg/ml concentration) with a non-relevant antibody-PAA complex for 1.5 hr at 4°C with shaking. Immunoprecipitation was done by incubating precleared lysates with the specific antibody-PAA complex for 1.5 hr at 4°C with shaking. Immune complexes were washed as follows: For non-stringent washings, beads were washed 5x with 1 ml lysis buffer (50mM HEPES pH7.3, 150 mM NaCl, 1% Triton x-100 , 10% Glycerol, 1.5mM $MgCl_2$, 5mM EGTA, 1mM Na_3Vo_4, 50mM NaF, supplemented with 100µg/ml leupeptin, 2mM PMSF, 25 units/ml Aprotinin.

For stringent washings, beads were washed once with 1 ml buffer (20mM Tris pH 7.4, 50mM NaCl, 1mM EDTA, 0.5% NP-40, 0.5% SDS, 0.5% deoxycholate) once with 1 ml RIPA buffer (10mM Tris pH 7.4, 150 mM NaCl, 1% NP-40, 1% deoxycholate, 0.1% SDS) once with 1 ml high salt buffer (2M NaCl, 10mM Tris pH 7.4, 1% NP-40, 0.5% deoxycholate) and finally again with 1 ml RIPA buffer. Beads were resuspended in 20µl 2x SDS-sample buffer with or without reducing agent, boiled for 5 min and analyzed on SDS-PAGE.

Immunohistochemical staining of frozen sections. Frozen sections were thawed and fixed in acetone at room temperature for 15 minutes, followed by a 5 minute rehydration in PBS. The sections were then blocked with PBS containing 2.5% skimmed milk and 50% normal mouse serum for 10 minutes at room temperature. The section was then washed once with PBS and the fluorescently labeled antibody (diluted in the above blocking solution) added, - anti-MUC1/ZD ZUM12D8 (labeled with AlexaFluor 488, Molecular Probes) to a final concentration of 50µg/ml, anti-MUC1/Y BOS6E6 (labeled with AlexaFluor 633, Molecular Probes) to a final concentration of 50µg/ml and anti-MUC1-tandem-repeat-array H23 (labeled with AlexaFluor 546, Molecular Probes) to a final concentration of 10µg/ml. The section was incubated with the antibodies for 90 minutes at room temperature in the dark, followed by 6 washes with PBS/0.2% Triton X-100. The slides were then air-dried and mounted with Gel/Mount (Biomeda Corp., Foster City, CA) and covered with a cover slip. The slides were analyzed by fluorescent microscopy or by confocal laser microscopy.

Fluorescent labeling of bacteria. Bacteria were labeled with the fluorescent dye PKH67/46 exactly as described by the manufacturer (Sigma, St Louis, MO).

Testing of binding of labeled bacteria to MUC1/ZD expressing cells. Cells were seeded onto poly-L–lysine coated cover slips placed into each well of a 24 well plate. At approximately 70% confluency, the cells were washed with PBS and fluorescently labeled bacteria, diluted in PBS, were added to the cells for one incubation at 25°C. The cells were then washed with PBS, fixed with 2% paraformaldehyde, and the cover slip inverted and mounted on a glass slide with Gel/Mount. The slides were analyzed by fluorescent microscopy or by confocal laser microscopy.

BODY-

RESULTS

A Tandem-Repeat-Array-Lacking MUC1 mRNA with a +1 Frameshifted 3' End

The splicing event that generates MUC1/Y (Fig. 1A 2), utilizes splice donor- and acceptor- sites located 5' and 3' to the central tandem repeat array, and splices out the central tandem repeat array and its immediate flanking sequences. The MUC1 open reading frame (ORF) downstream to the 3' splice acceptor site is, however, retained (Fig. 1A 2). An additional alternatively spliced tandem-repeat-array-lacking MUC1 mRNA identified by RT-PCR (see Methods), and termed here MUC1/ZD (Fig. 1A 3), utilizes an identical splice donor site to that used by MUC1/Y. The MUC1/ZD mRNA isoform could thus generate a protein identical at its amino terminal to the transmembrane MUC1/Y protein, including the MUC1 signal peptide. In contrast to MUC1/Y, the splice acceptor site utilized by MUC1/ZD is located 19 nucleotides downstream to that used by MUC1/Y (Fig. 1A3, Fig. 1B). As a consequence of alternate splice acceptor usage, the MUC1/ZD reading frame distal to the splice acceptor site is +1 frameshifted. The MUC1/ZD C-terminal unique region spans 43 amino acids, and is completely different to any other known MUC1 protein. In contrast to the transmembrane MUC1/REP and MUC1/Y proteins, it does not contain a transmembrane domain.

MUC1/ZD Protein Expression in Western Blots

The expected size of the MUC1/ZD mRNA is very close to that of the MUC1/Y mRNA species (only 19 nucleotides smaller), rendering the demonstration of MUC1/ZD expression by northern blot analysis difficult if not well nigh impossible. To circumvent this issue, we analysed MUC1/ZD expression at the protein level, using affinity purified anti-MUC1/ZD polyclonal antibodies. To investigate whether MUC1/ZD is at all expressed as a protein, western blots of tissue lysates prepared from human tumor samples were probed with antibodies generated against bacterial recombinant GST-MUC1/ZD protein. The expected size of the MUC1/ZD protein (73 amino acids), predicted that it should migrate in the region just above the 6kDa marker protein. Immunoreactive bands migrating with this molecular weight were observed in three out of the eight tissue samples analyzed (Fig. 2A, lanes 1, 4 and 7). Competing MUC1/ZD protein abrogated immunoreactivity (Fig.2C). Reprobing of the blot (previously probed with anti-MUC1/ZD antibodies, Fig. 2A) with anti-MUC1 cytoplasmic domain antibodies, demonstrated immunoreactive bands migrating between 25 and 30kDa (Fig. 2D, lanes 2 and 5, with lesser amounts observed in lanes 3 and 7). These bands represent the MUC1 cytoplasmic-domain-containing cleavage products generated by proteolytic cleavage of the MUC1/REP protein- presence of the 25-30kDa proteins reflects MUC1/REP expression. Expression of the MUC1/ZD protein did not go hand-in-hand with expression of the MUC1/REP protein and only one tissue sample (Fig. 2, lane 7) showed expression of both the MUC1/ZD and MUC1/REP proteins.

MUC1/ZD Protein Expression By Immunohistochemical Staining

In order to substantiate MUC1/ZD protein expression in human tissues, an immunohistochemical approach was adopted. Expression of the MUC1/ZD protein in breast tumor tissue was clearly demonstrated (Fig. 3A). Addition of the immunizing MUC1/ZD protein abrogated immunohistochemical reactivity (Fig. 3B), thereby confirming the specificity of antibody staining. In line with these findings, addition of GST protein itself had no effect on the immunoreactivity (Fig. 3C).

Taken together, the western blot and immunohistochemical analyses demonstrated MUC1/ZD expression at the protein level in human tissues.

The MUC1/ZD Protein Exists As a Disulfide Linked Oligomer

The unique MUC1/ZD 43 amino acid C-terminal domain contains 3 cysteine residues (Fig. 1B, highlighted amino acid residues), raising the possibility that the MUC1/ZD protein may form intra- and/or inter-molecular disulfide bonds. To test for this, lysate of a tissue known to express the MUC1/ZD protein, was run under both reducing and non-reducing conditions and then submitted to a western blot analysis. Under disulfide-bond-reducing-conditions the tissue lysate displayed, as expected, an immunoreactive band migrating just above the 6kDa marker protein (Fig. 4A, lane 1). When non-reducing conditions were employed, the low molecular weight 6-7kDa species was no longer seen (Fig. 4A, lane 2), and in its place a distinct immunoreactive band

migrating at about 64kDa was readily observed.

To confirm that the 64kDa band represents the MUC1/ZD protein, proteins extracted from two tumor tissue lysates, one a known MUC1/ZD positive sample (as determined by western blotting under reducing conditions) and the other a MUC1/ZD- negative sample, were run under identical non-reducing gel electrophoretic conditions. The proteins migrating in the 64kDa region were eluted from the gel, radio-iodinated and equivalent levels of radioactivity obtained from both samples were subjected to immunoprecipitation using anti-MUC1/ZD antibodies. This revealed a 64kDa band that appeared exclusively in the MUC1/ZD expressing sample (Fig. 4B, lane 1)-no imunoprecipitating bands were seen in the MUC1/ZD negative sample.

Further support for the disulfide bonded character of the 64kDa MUC1/ZD species was obtained by running a MUC1/ZD positive tumor tissue lysate in two dimensions. In the first instance, two identical lanes were run in the first dimension, under non-reducing conditions- following electrophoresis, one lane was taken and placed *horizontally* on a new gel and run in the second dimension, once again under *non-reducing* conditions. The second identical lane was soaked in reducing sample buffer and run on a new gel under *reducing* conditions. The two gels were then blotted and subjected to anti-MUC1/ZD probing. In the event that the 64kDa anti-MUC1/ZD immunoreactive species is not disulfide bonded, then it should appear in both western blots at a position located on the diagonal of the 2-dimensional blot. On the other hand, if the 64kDa anti-MUC1/ZD immunoreactive species is a disulfide bonded oligomer of a 6-7kDa MUC1/ZD protein subunit, it should then appear on the diagonal of the 2-dimensional blot *only* in the non-reduced second gel. In the *reduced* 2nd-dimension gel it would appear at a location off the diagonal, and correspond to migration of a 6-7kDa protein. This was indeed the case. The spots designated by the open arrowhead in Fig. 4C-a represents the oligomeric MUC1/ZD protein complex that following reduction migrates with a molecular weight of 6-7kDa (4C-b). Note that in these experiments in order to get a good resolution within the low molecular weight region a high percentage acrylamide gel was used. This dense acrylamide gel results in the piling up of proteins above 64kDa that could well explain the non-specific immunoreactive spots (see Figure 4Ca and 4Cb,) representing proteins with molecular weights higher than 64 kDa. Because of this concern we reanalyzed MUC1/ZD positive and negative samples on a lower percentage acrylamide gel, under non-reducing conditions, and performed a long electrophoretic run in order to give a good separation in the region just below and above 60kDa. This immunoblot showed the 64kDa MUC1/ZD complex is present solely in the MUC1/ZD positive sample (data not shown). Taken together, these experiments support the notion that the MUC1/ZD protein exists as an oligomeric complex composed of MUC1/ZD protein subunits that are disulfide bonded to each other.

Generation of Anti-MUC1/ZD Monoclonal Antibodies

As the MUC1/ZD protein is in fact a totally novel protein derived from the MUC1 gene, monoclonal antibody reagents were generated that are specific for this novel MUC1 protein. These antibodies were then used to compare MUC1/ZD expression with expression of the MUC1/REP protein, as well as to assess MUC1/ZD protein levels in body fluids.

Mice were immunized with a GST-MUC1/ZD fusion protein and the hybridoma supernatants screened for their immunoreactivity against both the GST-MUC1/ZD fusion protein and, in this case, non-relevant GST-MUC1/N-terminal protein. Monoclonal antibodies reacting positively towards the GST-MUC1/ZD protein and non-reactive with the GST-MUC1/ N-terminal protein represent MUC1/ZD-specific monoclonal antibodies. Using this strategy two particularly reactive clones, ZUM12D8 and ZUM7E7, were obtained and chosen for further study. These hybridomas secrete immunoglobulins (both Ig-gamma1 subtypes) that react only with the MUC1/ZD protein (Fig. 5A)- they were non-reactive towards the MUC1/Y protein (Fig. 5B) and did not react with the N-terminal portion of the MUC1 protein (Fig. 5C). This indicated that both ZUM12D8 and ZUM7E7 recognize the unique C-terminal portion of the MUC1/ZD protein and are thus specific for the MUC1/ZD protein.

An ELISA Assay for Detecting MUC1/ZD Protein

The peptide TTKSCRETFLK present in the C-terminal forty-three MUC1/ZD-unique amino acids is highly hydrophilic, suggesting that it may be an immunogenic site located on the surface of the MUC1/ZD protein.

This peptide was therefore tested to see whether it could inhibit the immunoreactivity of MUC1/ZD specific monoclonal antibodies. Whereas addition of this peptide competed out the immunoreactivity of the ZUM7E7 antibody, as assayed by western blotting against recombinant MUC1/ZD, it was without any effect on the ZUM12D8 monoclonal antibody (data not shown). This suggests that the two MUC1/ZD specific monoclonal antibodies recognize distinct epitopes, endorsing the development of a sandwich ELISA assay for MUC1/ZD protein detection. ELISA formats were established in which the capture antibody consisted of ZUM7E7 and the detecting antibody comprised biotinylated ZUM12D8. This assay could detect MUC1/ZD down to a protein concentration of approximately 50 picograms per milliliter, and no cross-reactivity with the MUC1/Y protein or any other MUC1 protein was observed.

Using this assay, we screened sera from four normal individuals as well as sera from ten breast cancer patients- no MUC1/ZD protein could be detected. Effusions obtained by aspirating fluids from the sites of breast lumpectomies were next examined for the presence of the MUC1/ZD protein. Eight separate effusion samples originating from a total of seven patients were investigated. MUC1/ZD protein was found in the effusions of only one patient, at levels corresponding to about 2 nanogram/ml (40 fold higher than the detection limit of the assay). Two separate samples aspirated at different time intervals (3 days and 7 days) following the lumpectomy operation, were obtained from this same patient. Both samples were positive for the MUC1/ZD protein, with slightly higher levels present in the effusion sample obtained at the earlier post-lumpectomy time-point.

Immunohistochemical Analysis of MUC1/ZD Expression with Monoclonal Antibodies

Preliminary immunohistochemical studies demonstrated that ZUM12D8 could detect the MUC1/ZD protein in tissue sections. As the best immunoreactivity, by far, was obtained with frozen tissue sections that had undergone acetone fixation, expression of the MUC1/ZD protein as well as that of additional MUC1 protein isoforms was assessed in such sections.

Substantial expression of the *MUC1* gene in skin, and in sebaceous gland tumors in particular, has been recently documented (39). This provided the rationale for looking at *MUC1* gene expression, and specifically at the MUC1/ZD protein, in this tissue. To set the stage for future work involving the simultaneous detection of several MUC1 protein isoforms, purified ZUM12D8 anti-MUC1/ZD monoclonal antibody was fluorescently labeled and direct immunofluorescence on frozen tissues assessed.

MUC1/ZD immunoreactivity was readily detected in frozen sections of skin using the green fluorescently labeled anti-MUC1/ZD ZUM12D8 monoclonal antibody (see Figure 6A, D and E and compare with the hematoxylin stained consecutive sections C, F and I). The anti-MUC1/ZD immunoreactivity was limited to epithelial cells comprising the sebaceous glands, hair follicles and the epithelial cell layers forming the skin surface- essentially all immunoreactivity was competed out by adding the immunizing MUC1/ZD protein, hence validating the specificity of immunoreactivity (compare panels A, D and E with panels B, E and H). Immunoreactivity could not be observed in fibroblasts embedded within the connective tissue nor in the connective tissue itself.

The generation and utilization of monoclonal antibodies specific for the MUC1/ZD protein, demonstrated expression of this protein in human tissue. Having established MUC1/ZD protein expression in skin-tissue-epithelial cells, we next wished to compare the expression of the MUC1/ZD protein with that of the classical transmembrane tandem-repeat-array containing protein, MUC1/REP. A frozen section of a skin tissue sample was reacted simultaneously with red fluorescently labeled anti-MUC1 tandem-repeat array H23 antibodies and green fluorescently labeled anti-MUC1/ZD ZUM12D8 antibodies. This double-staining procedure demonstrated expression of both MUC1 proteins in epithelial cells comprising the sebaceous gland (Figure 7). Some of the labeled cells displayed expression of only one of the two isoforms (compare Figures 7A and 7B). Peripheral epithelial cells appeared to express primarily the MUC1/ZD protein (green fluorescence in Figure 7A), whereas cells more centrally located displayed red fluorescence signifying MUC1/REP expression (Figure 7B). Additional information obtained from a computer-generated overlay of the two fluorescent signals, demonstrated that cells located at the interface between the peripheral and more centrally located epithelial cells, expressed almost equal levels of the MUC1/ZD and MUC1/REP protein isoforms (yellow fluorescence in Figure 7C). Higher magnification analyses of those cells expressing both MUC1 isoforms

indicated that the MUC1/REP protein was located almost exclusively on the cell surface, as expected of a membrane-bound protein (red fluorescence, Figure 7E). In contrast, the MUC1/ZD protein could be seen both on the cell surface as well as localizing in interstitial spaces (green fluorescence, Figure 7D). A computer-generated merge confirmed this assessment (Figure 7F, yellow color designates regions expressing both MUC1/REP and MUC1/ZD whereas the green color indicates MUC1/ZD expression alone).

Function of the MUC1/ZD Protein in Bacterial Binding to Cells- To gain insight into the possible biological function of the MUC1/ZD protein, a *tblastn* homology search was conducted. Proteins showing similarity with the complete gamut of MUC1/ZD amino acid sequence, ranging from its N- to C-termini, could not be identified. However a selected group of proteins did display limited sequence similarity to the unique region of the MUC1/ZD protein, the C-terminal 43 amino acids (Figure 8). This group comprised CD14 (rat monocyte differentiation antigen), defensin-like peptide 1, a plant defensin and the anaphylatoxin domain signature. Notably for three of the proteins identified- CD14, plant defensin and the anaphylatoxin domain signature- the region of similarity encompasses the three-cysteine residues of the MUC1/ZD protein. The most extensive similarity was found between MUC1/ZD and CD14 (Figure 8A), a protein known to bind to and present bacterial cell surface components to the cell, thereby allowing appropriate cell-protective action to be initiated. All four proteins in this group are involved in safeguarding the cell against bacterial or/and fungal invasion (40-44). The classical perspective of tandem-repeat-array-containing mucin proteins is that they may act as cell-protective agents. Viewed in this light, the shared cell-protection denominator of that is common to MUC1/ZD and the four identified proteins is all the more intriguing.

To investigate whether the MUC1/ZD protein harbors direct bacteriocidal activity, as do the defensin proteins, recombinant MUC1/ZD protein was purified and added to bacterial cultures- neither bacteriostatic nor bacteriocidal activity was observed.

To see whether MUC1/ZD could act in a similar fashion to CD14, by recognizing and binding to bacterial cell-surface components, a simple initial experiment was conducted. Recombinant MUC1/ZD protein was fluorescently labeled and added to unlabeled bacteria. To avoid the possibility of non-specific MUC1/ZD sticking to the bacteria, incubation was performed in a serum-containing blocking solution, which provided high concentrations of non-relevant competing proteins. Several bacteria were fluorescently labeled (Figure 9A), suggesting that the MUC1/ZD protein could indeed bind to bacteria.

To provide more clear-cut evidence for an interaction between MUC1/ZD and bacteria, we resorted to stable cell transfectants expressing MUC1/ZD. As a first step in this direction stable mouse 3T3 cell transfectants expressing MUC1/ZD were generated. The choice of these cells rested on experiments showing that parental 3T3 cells, which are of fibroblastic lineage, did not bind bacteria. Analysis of transfectants with the MUC1/ZD specific monoclonal antibody ZUM12D8 followed by fluorescent activated cell sorting (FACS) analysis, confirmed that they expressed the MUC1/ZD protein (Figure 9B).

Reaction of fluorescently labeled bacteria (seen as red fluorescence) with the stable MUC1/ZD-expressing 3T3 transfectant (MUC1/ZD-3T3) clearly showed bacterial binding to the cells (Figure 9C I). Addition of excess unlabeled bacteria abrogated all cell binding (Figure 9C II), suggesting specificity of interaction of bacteria with the MUC1/ZD-3T3 transfectant. Parental non-MUC1/ZD expressing 3T3 cells did not sustain bacterial binding (Figure 9C III).

Binding of bacteria to MUC1/ZD-expressing cells compared to the absence of bacterial binding to 3T3 cells that do not express MUC1/ZD, consolidated the thesis that the MUC1/ZD protein mediates this binding.

BODY-

DISCUSSION

The *MUC1* gene has captured the interest of many research groups. Its protein products, primarily those containing tandem repeats, have been intensively investigated and more than one thousand publications relate to the *MUC1* gene and its proteins. We describe here a novel MUC1 protein, MUC1/ZD, that is derived from the *MUC1* gene. Its novelty is typified by two characteristics- firstly, it does not contain the hallmark tandem-repeat-array domain distinctive of mucin proteins and secondly the complete C-terminal segment is a novel peptide sequence, absent from all other known MUC1 protein isoforms.

Generation of the MUC1/ZD protein results from an alternative splicing event, which deletes the central tandem repeat array and effects a +1 change in reading frame immediately downstream to the splice acceptor site. Although there are instances when frameshifting engendered by alternative splicing does occur in eucaryotic cells (47,48), this mechanism is a rare event in such cells. It is much more common in viruses which in order to expand the encoding capabilities of a limited genome often invoke alternative splices with accompanying reading frameshifts (49). It is of interest to note that of all the *MUC1* genes documented to date, only the simian (50)(GenBank™ accession no. AF176947) and porcine (GenBank™ accession no. AY243508) (in addition to human) *MUC1* genes have the potential to produce a MUC1/ZD-like protein, generated by splice sites located upstream and downstream to a central tandem repeat array. Indeed, the mouse *MUC1* gene transcribes an mRNA in which the N-terminal signal peptide coding sequence leads almost directly into the tandem-repeat–array (51), thereby effectively proscribing the formation of a MUC1/ZD-like protein. This contrasts with the human (and simian and porcine) *MUC1* genes, which comprise a significant region that is located between the N-terminal signal peptide and the central tandem-repeat-array. It thus appears that rather than being strongly conserved the *MUC1* gene is, in one sense, rapidly evolving and in its passage from mouse to man the 5' end of MUC1 has been elaborated with the capability of generating novel and species-specific proteins. On the other hand, the C-terminal domains of the MUC1/REP protein do retain high inter-species conservation- this is especially true of the highly conserved cytoplasmic and transmembrane domains as well as the sequences immediately N-terminal to the transmembrane domain (52). These MUC1 domains are being implicated more and more in signal transducing functions (24-27) and as such there may have been strong evolutionary constraints on conservation of these regions of the MUC1 protein.

The tandem-repeat-array containing MUC1 protein is known to be highly expressed in breast cancer cells as well as in epithelial cells of the lactating breast and at lower levels in resting breast tissue, other epithelial tissues and in cells of the immune system such as activated T cells, B cells and dendritic cells (53-55). Moreover its expression has also been demonstrated in epithelial cells comprising skin tissue (39). Here we show that expression of the novel MUC1/ZD protein is unequivocal- we have demonstrated its expression using affinity purified polyclonal antibodies and MUC1/ZD monospecific monoclonal antibodies by five independent modalities including western blotting, immunohistochemistry, immunoblotting, immunoprecipitation and ELISA assays. Its expression was observed both in cancer tissue and in epithelial cells comprising the epithelial skin layers and sebaceous glands. Interestingly, its expression did not correlate with that of the MUC1/REP protein. This was particularly evident in epithelial cells of the sebaceous gland. Likewise, some tumors expressed primarily MUC1/ZD with little MUC1/REP expression and vice versa. Some cells did however express both the MUC1/REP and MUC1/ZD proteins and this was clearly evident in double immunohistochemical stainings done on skin tissue sections. These results suggest that the choice as to which MUC1 mRNA species is expressed occurs at the level of alternative splicing, and that the cell may, depending on its specific needs at any particular time, require expression of one or the other, or any combination, of the MUC1 protein isoforms. This may depend on the differentiation status of the cell or on whether extracellular changes have taken place that lead to expression of a particular MUC1 isoform or a particular blend of MUC1 protein isoforms.

From its protein makeup, one would expect MUC1/ZD to be a secreted protein- it lacks a transmembrane domain yet comprises an N-terminal signal peptide (56). Double labeling immunohistochemical analyses

indicated, however, that MUC1/ZD was sited both at the cell membrane as well as in its close proximity. In line with these results we could barely detect MUC1/ZD protein in the culture medium of transfectants that clearly expressed, as analyzed by fluorescent cell sorting, MUC1/ZD. Furthermore, out of a total of eight breast effusions and fourteen serum samples analyzed we detected this protein in only two breast effusion samples - notably both positive effusion samples were from the same patient who likely had very high MUC1/ZD expression levels. These results conform to those of the immunohistochemical analyses and suggest that despite the fact that MUC1/ZD is lacking a transmembrane domain, it is situated close to the cell membrane. We do not know what is maintaining the "transmembrane-less" MUC1/ZD protein at the cell surface. It could be that it is interacting with (modified) proteoglycans present close to the cell membrane. Alternatively it may be that the MUC1/ZD C-terminal leucine-tryptophan-tryptophan-tyrosine hydrophobic sequence (LWWY), especially when appearing in a MUC1/ZD oligomeric complex, is sufficient to loosely attach the MUC1/ZD protein to the cell membrane.

Besides its unexpected cellular location, the MUC1/ZD protein is also special in the sense that it presents as an oligomeric protein complex likely composed of monomeric MUC1/ZD subunits that interact with each other by disulfide bonds (Figure 4A and 4B). Indeed MUC1/ZD comprises three cysteine residues containing reactive sulfhydryl groups that can potentially form covalent disulfide intermolecular disulfide bonds. Examples are extant in the literature of secreted ligands that act as disulfide bonded dimers consisting of either homo- or heterodimers (57,58) - oligomers consisting of more than two subunits are also documented although they are less common. The MUC1/ZD oligomer is quite obviously not a dimeric protein and its molecular size suggests that it comprises about ten MUC1/ZD monomeric subunits. Although it is theoretically possible that the MUC1/ZD-oligomer comprises heterologous proteins covalently bonded to MUC1/ZD, we do not have any evidence to date to support such a model. On the contrary, work with bacterial recombinant MUC1/ZD protein demonstrates that the monomeric subunits form oligomers by covalently binding to each other (data not shown). Because of its three cysteine residues, one could envisage that each MUC1/ZD subunit employs two of its three cysteine residues to bind to an adjacent MUC1/ZD monomer to form a dimer containing two disulfide bonds- the vacant third cysteine residue present on each of the dimer partners may be used to form a disulfide bond with adjacent MUC1/ZD dimers. If a string-like oligomer were formed, this would lead to MUC1/ZD termini that have unoccupied, highly reactive, free cysteine residues, one at each side of the oligomeric string. A more plausible model would thus be that the MUC1/ZD termini also form a disulfide bond via their vacant third cysteine residues, thus fashioning a ring-like structure consisting of ten disulfide-linked MUC1/ZD monomers. Precedence exists for a circular/ring-like protein- indeed the bacteriocidal protein θ-defensin has such a ring-like structure, formed in part by disulfide bonds (3,59,60) . The rather special oligomeric configuration of the MUC1/ZD protein may thus be of significance vis-à-vis its cellular function.

What indeed is the function of the novel MUC1/ZD protein described here? A *tblastn* search revealed that the best fits were seen between MUC1/ZD and a group of proteins that are known to function as parts of the innate immune system, either directly by acting as bacteriocidic agents or indirectly by participating in recognition of bacterial components and their presentation to the cell (40,42-44) . Although by no means extensive, this similarity is noteworthy in that the homologous regions map to that part of MUC1/ZD that comprises the cysteine residues. For example, all MUC1/ZD cysteine residues align with counterpart cysteines present in the CD14, plant defensin and anaphylatoxin proteins.

Experimental work showed that whereas the MUC1/ZD protein possessed neither intrinsic bacteriocidal nor bacteriostatic activity, it did participate in binding to bacteria and presenting them to the cell. In these respects, MUC1/ZD resembled CD14, the innate immunity protein. MUC1/ZD shared the best homology with the N-terminal part of CD14- it extended over 44 amino acids showing 29% identity and 54% similarity. For recognizing bacterial cell surface components, the N-terminal region of CD14 is indispensable (61), and it has been proposed that this region of CD14 acts autonomously in the recognition process (62). Till now, the CD14 protein had the distinction of having no known homologous proteins that act in a similar manner- indeed a tblastn search with the N-terminal part of CD14 does not reveal any homologous proteins, besides that of

MUC1/ZD. The similarity between MUC1/ZD and CD14 both at the primary amino acid level as well as at the functional level is, for this reason, that more remarkable.

CD14 is rather promiscuous in its recognition of bacterial cell surface components- indeed it binds both lipopolysaccharride (LPS) as well as proteoglycan cell surface components (63,64). The bacterial components recognized by MUC1/ZD have not been characterized and this issue remains beyond the scope of the present investigation. However, we have here seen binding of recombinant MUC1/ZD to Staphylococcus Oxford, a gram-positive bacterium as well as, in the cell binding experiments, to Pseudomonas Aeruginosa, a gram-negative bacterium. It would therefore appear that the analogy between CD14 and MUC1/ZD is even more far-reaching in that MUC1/ZD, like CD14, can bind to components present on both gram-positive and gram-negative bacteria.

In a sense we have come full-circle regarding the proposed function of the *MUC1* gene and its protein products. Our results support the classical view of the mucin-like proteins and, in our context, the protein products of the *MUC1* gene, as protecting the cell against untoward changes in the extracellular environment be they physico-chemical or/and biological changes. What our investigations may be highlighting is that whereas the primary function of the *MUC* genes is to generate proteins that participate in cell protection, the specific *MUC*-gene-derived proteins themselves are not necessarily obliged to comprise a mucinous tandem repeat array. They are, however, obliged to participate in some fashion in cell protection. In our specific case this would be bacterial recognition, presentation to the cell and initiation of cell signaling achieved by, at the very least, cooperation between the MUC1 isoforms MUC1/ZD and MUC1/REP. The primary function of the *MUC1* gene may thus be in cell protection and it is implementing this role by generating, via alternative splicing, a number of different proteins all of which function in order to accomplish this major objective.

It is clear that the MUC1 proteins are highly upregulated in malignant breast cancer cells (65). How then are we to reconcile the proposed function of MUC1 proteins in cell protection with their increased expression in the breast cancer cell? We posit that indeed MUC1 expression, per se, may play no major causative role in the transformation of the normal breast epithelial cell into its opposite number, the malignant cell. The rapidly proliferating breast malignant epithelial cell is likely simply mimicking the feature of increased MUC1 protein expression from, for example, its normal counterpart the proliferating normal epithelial cell of the lactating breast, in which MUC1 expression is also highly upregulated (66). It is likely that the function of increased MUC1 expression in the lactating epithelial cell is for cell protection. This proposed scheme of events by no means excludes the very real possibility of utilizing the MUC1 proteins on the cancerous cell as targets to kill such cells. Furthermore, such a line of reasoning underscores the importance of unraveling the ways the MUC1 proteins function. Such knowledge may well be used in the future to subjugate pathways taken by the MUC1 proteins in order to effect eradication of the cancer cell.

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LEGENDS TO FIGURES

Figure 1. Scheme of different MUC1 isoforms.

A. The splicing events that generate the various MUC1 mRNA isoforms are presented. MUC1/ZD mRNA is generated by alternative splicing that utilizes the same splice donor site as that utilized by the MUC1/Y isoform- the MUC1/ZD splice acceptor site (S.A.*) is located 19 nucleotides downstream to that used by MUC1/Y (see Fig. 1B for the exact locations of the splice sites). The alternate splice acceptor site S.A.*, effects a reading frameshift and this is depicted by the stippled exons downstream to this site.

B. The MUC1/ZD nucleotide and protein sequence. Numbering of the nucleotide sequence appears to the left and amino acid numbering appears to the right of the figure. The site of the splice event that generates the MUC1/ZD mRNA and protein is indicated by the downward facing arrow just C-terminal to amino acid number 53 (after the amino acid sequence EKNA). Cleavage of the signal peptide is predicted to occur between Gly^{23} and Ser^{24} (indicated by the upward facing red arrow)- the MUC1/ZD protein will thus have thirty N-terminal amino acid sequences (stretching from Ser^{24} to Ala^{53}) in common with the other MUC1 isoforms. The subsequent forty-three C-terminal amino acids are unique to the MUC1/ZD protein.

A. MUC1 cDNA forms

B. MUC1/ZD Sequence

"KOZAK"	Met

```
1                          gaatctgttctgcccctccccacccatttcaccaccaccatg
                                               Signal peptide          M   1
61   acaccgggcacccagtctccttttcttcctgctgctgctcctcacagtgcttacagttgtt
      T   P   G   T   Q   S   P   F   F   L   L   L   L   L   T   V   L   T   V   V  21
121  acaggttctggtcatgcaagctctaccccaggtggagaaaaggagacttcggctacccag
      T   G   S   G   H   A   S   S   T   P   G   G   E   K   E   T   S   A   T   Q  41
                                                              Splice Donor
181  agaagttcagtgcccagctctactgagaagaatgctgtgagtatg....................
      R   S   S   V   P   S   S   T   E   K   N   A
                            Splice Acceptor [MUC1/Y]   Splice Acceptor  [MUC1/ZD]
..........................................cacatttcaaacctccagtttaattcctctctggaagatccc
                                                                            I   P
agcaccgactactaccaagagctgcagagagacatttctgaaatgttttttgcagatttat
 A   P   T   T   T   K   S   C   R   E   T   F   L   K   C   F   C   R   F   I

aaacaagggggttttctgggcctctccaatattaagttcaggccaggatctgtggtggta
 N   K   G   V   F   W   A   S   P   I   L   S   S   G   Q   D   L   W   W   Y
caattga
 N   *
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Figure 2. Immunoblotting detection of the MUC1/ZD protein in tissue lysates.

Bacterial recombinant MUC1/ZD protein (A) and protein lysates from human carcinoma tissues (B, C and D) were resolved by 12.5% SDS-PAGE, electro-transferred to a nitrocellulose membrane, probed with either affinity purified rabbit polyclonal anti-MUC1/ZD antibodies (A, B and C) or affinity purified antibodies directed against the MUC1 cytoplasmic domain. Detection was achieved by enzyme-linked goat anti-rabbit antibodies followed by chemiluminescence (ECL) as described in Experimental Procedures. To determine anti-MUC1/ZD specificity either GST-MUC1/ZD recombinant protein (C, GST-MUC1/ZD comp.) or a 12-mer peptide derived from MUC1/ZD unique region (C, peptide comp.) were added at a 100 fold molar excess to the anti-MUC1/ZD antibody preparation. Human carcinoma tissues were derived either from colon/rectal lesions (lanes 1 and 4, panels B and D; and lanes 2 and 3 in panel C) or from breast tumors (lanes 2, 3, 5, 6, 7 and 8, panels B and D; and lane 1 in panel C).
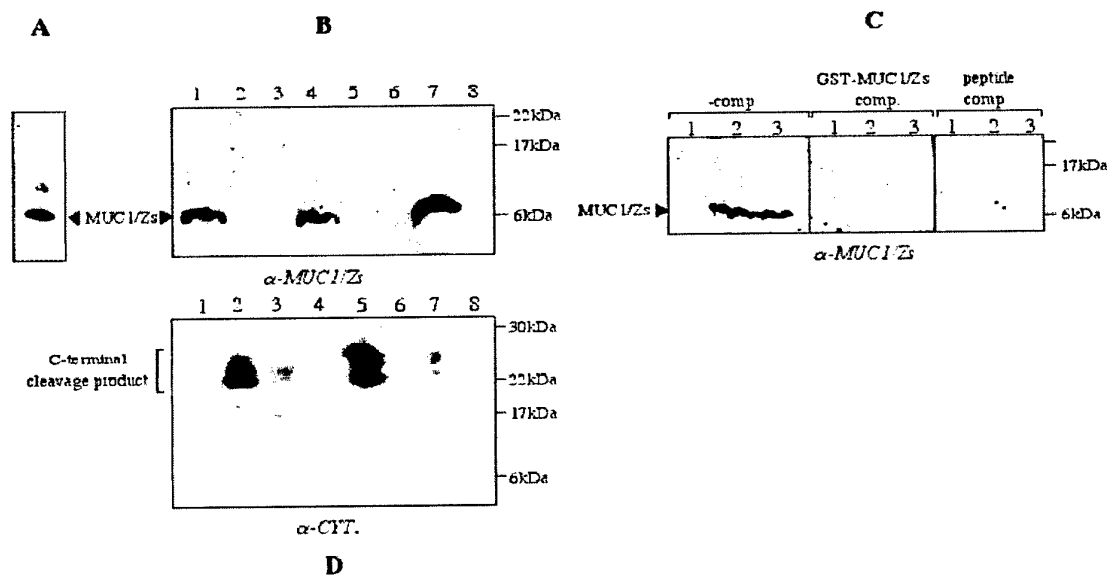
Figure 3. Immunohistochemical analysis of breast tumor tissue with anti-MUC1/ZD antibodies. Paraffin embedded sections from human breast cancer tissue were immunostained with affinity purified anti-MUC1/ZD polyclonal antibodies **(A)**. 100 fold molar access of either recombinant bacterial MUC1/ZD **(B)** or recombinant MUC1 N-terminal domain **(C)** were added to the antibody solution prior to the incubation with the tissue sections. Panels A, B and C- 200-fold magnification, Panels A', B' and C'- 1000-fold magnification. The brown staining in panels (A) and (C) represents expression of the MUC1/ZD protein.
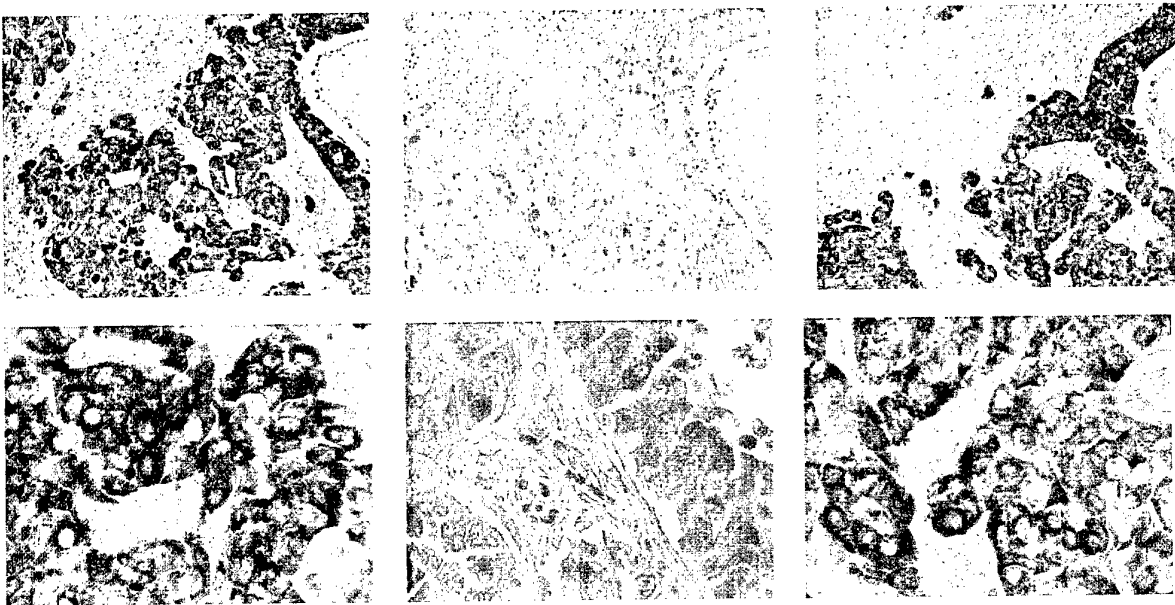
Figure 4. The MUC1/ZD protein exists as a disulfide-linked oligomer. (A) Protein lysate from a human carcinoma tissue that expresses MUC1/ZD was resolved on 12.5% SDS-PAGE either under reducing (lane 1) or non-reducing (lane 2) conditions and analyzed by western blotting using affinity purified anti-MUC1/ZD polyclonal antibodies. **(B)** Human carcinoma tissue that expresses MUC1/ZD and a non-expressing human carcinoma tissue were resolved on 10% SDS-PAGE under non-reducing conditions. Subsequently, the 60kDa region was excised from the gel, proteins eluted, [125]I labeled and immunoprecipitated with affinity purified anti-MUC1/ZD polyclonal antibodies. The labeled precipitating proteins were analyzed on 12.5% SDS-PAGE under non-reducing conditions. **(C)** Lysate from human carcinoma tissue that expresses MUC1/ZD was analyzed by diagonal gel electrophoresis. The first dimension was resolved under non-reducing conditions [1' (-)]where as the second dimension was performed either under non-reducing conditions [upper panel , 2'(-)]or under reducing conditions [lower panel , 2'(+)]. Subsequently, the gel was analyzed by western blotting using affinity purified anti-MUC1/ZD polyclonal antibodies. The open arrow (in panel C, a) indicates the non-reduced MUC1/ZD complex. The black arrow (in panel C, b) indicates the reduced MUC1/ZD monomer. The dashed circle (in panel C, b) indicates the expected location of the MUC1/ZD complex.
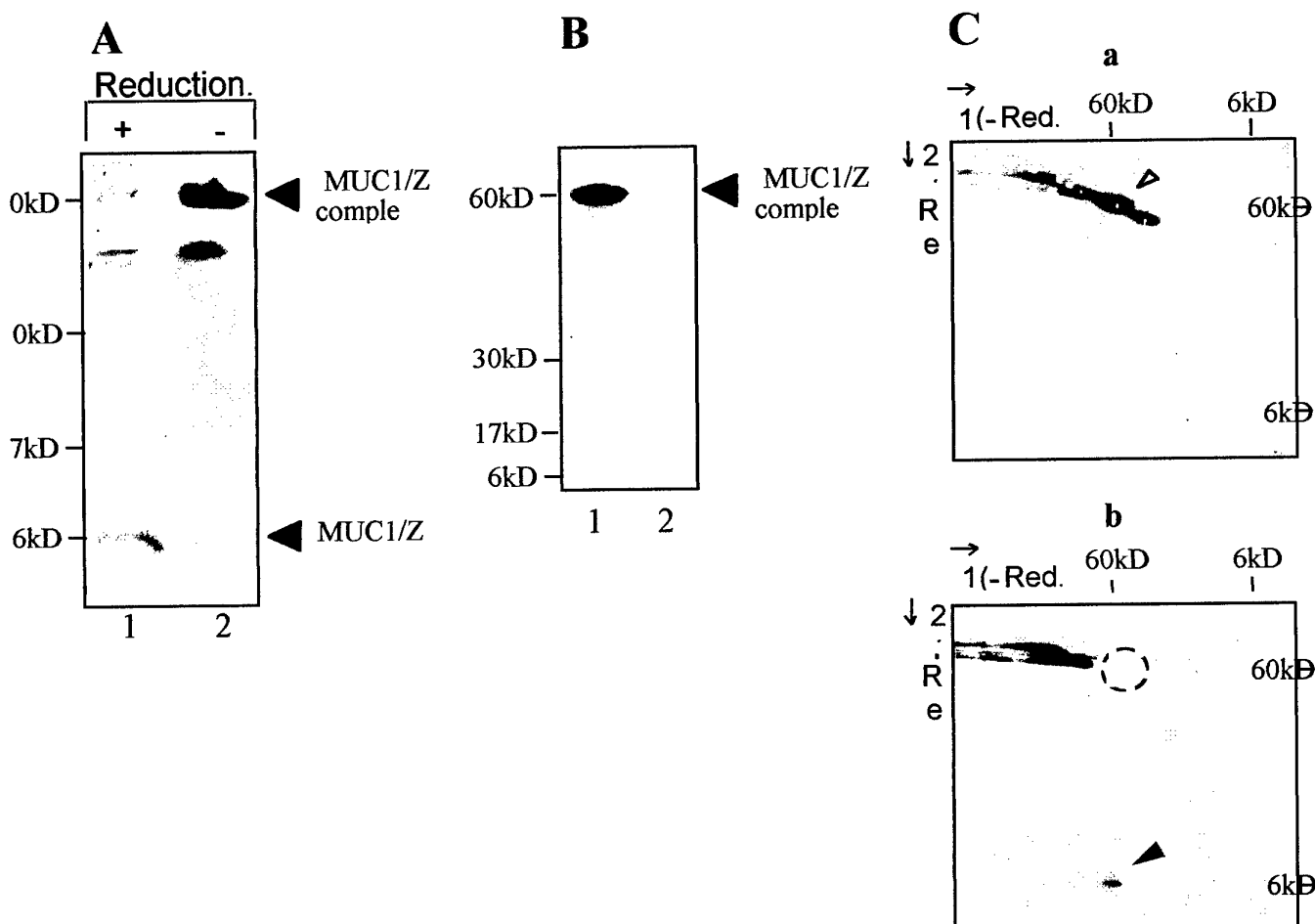
Figure 5. Specificity of the monoclonal antibodies ZUM12D8 and ZUM7E7 for MUC1/ZD protein.
Twenty nanograms per lane of MUC1/ZD protein (panel A), DHFR-MUC1/Yex (the extracellular domain of
MUC1/Y fused to the C-terminus of the non-relevant bacterial DHFR protein, panel B) and GST-N-terminal
MUC1/REP (the N-terminal sequences of MUC1/REP extending from the site of signal peptide cleavage till
the tandem repeat array fused to the C-terminus of GST, panel C) were resolved on 12% SDS-PAGE, blotted
and probed with: MUC1/ZD specific monoclonal antibody ZUM12D8 (lanes1), MUC1/ZD specific
monoclonal antibody ZUM7E7 (lanes 2), MUC1/Y specific monoclonal antibody BOS6E6 (lanes 3). Bound
mouse monoclonal antibodies were detected by enzyme-linked anti-mouse antibodies followed by
chemiluminescence (ECL). The upper and lower arrows indicate the positions of marker proteins migrating
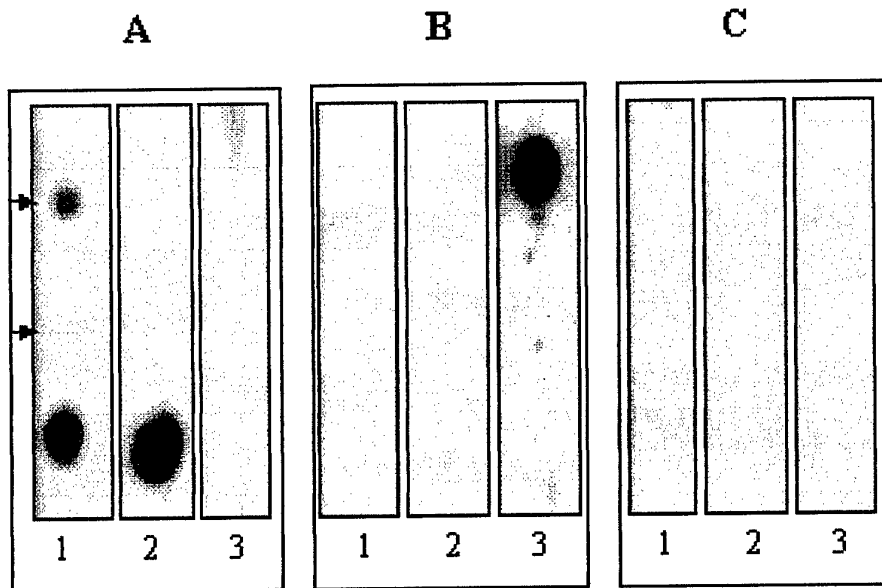with molecular masses of 30 and 17kDa, respectively.

Figure 6. Immunohistochemistry of skin tissue sections with green fluorescently labeled anti-MUC1/ZD specific monoclonal antibody ZUM12D8. Frozen sections of skin tissue were thawed, fixed in acetone, rehydrated in PBS, blocked in PBS containing 2.5% skimmed milk and 50% normal mouse serum and reacted with a solution of PBS containing 40□g/ml of green fluorescently labeled (Molecular probes, Alexa 488) anti-MUC1/ZD monoclonal antibody ZUM12D8 and 50% normal mouse serum. This was followed by 4-6 washes with PBS/0.2% TritonX-100. The slides were then dried, mounted and observed by confocal-laser microscopy. Panels A, D and G represent different sections of skin tissue analyzed as above without any further additions. Panels B, E and H are serial sections successive to those appearing in A, D and G respectively, and were reacted as for A, D and G except for the addition of competing excess MUC1/ZD protein to the labeled antibody reagent. Panels C, F and I are also serial sections successive to those appearing in A, D and G respectively and were subjected to eosin/hematoxylin staining. Note the intense staining in A, D and G of the epithelial cells forming the sebaceous glands and the surface skin layers- no staining was observed with the fibroblasts or with any components of the connective tissue. All immunoreactivity was competed out by addition of competing soluble MUC1/ZD protein (panels B, E and H).

Epithelial cells of hair follicle

Epithelial cells forming sebaceous gland

Fibroblasts in connective tissue

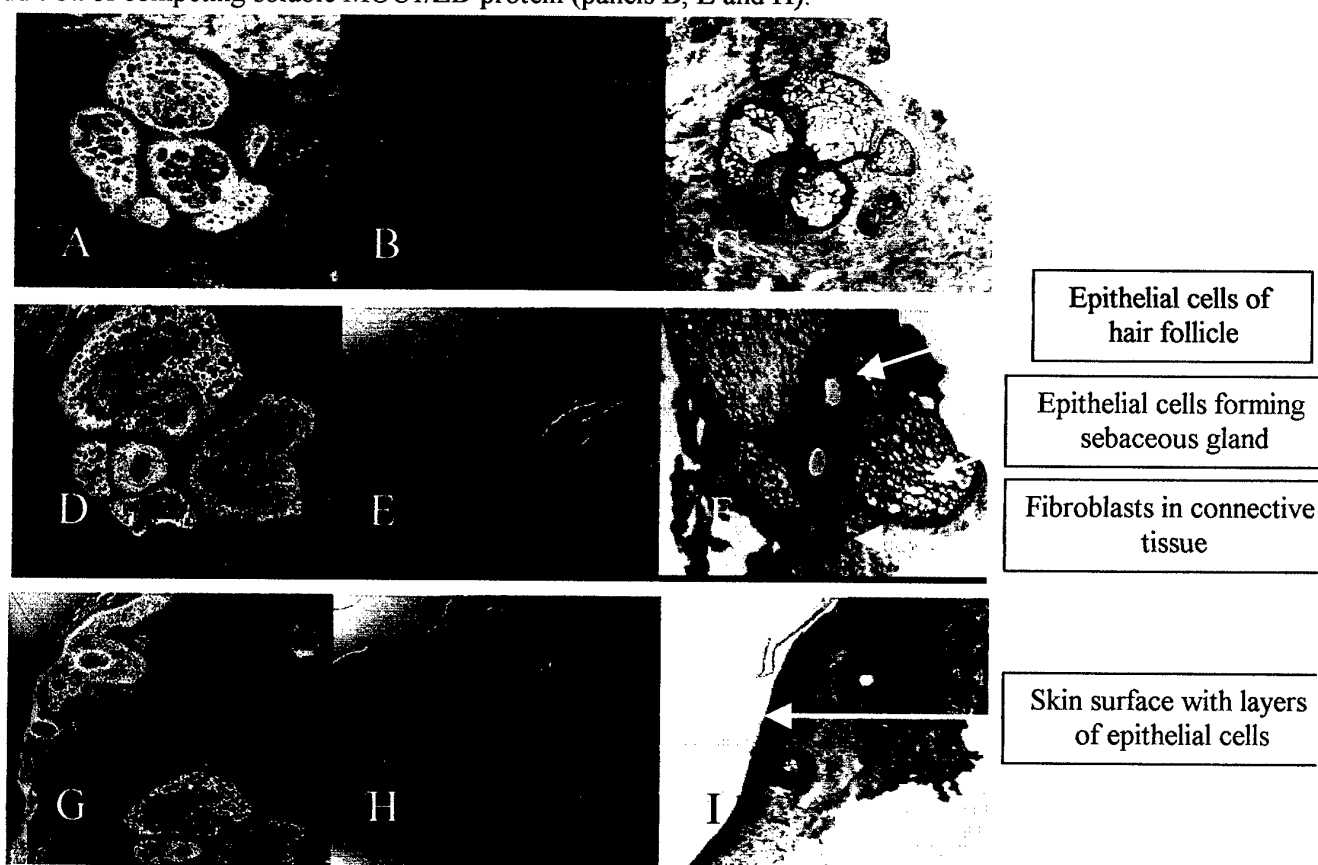Skin surface with layers of epithelial cells

Figure 7. Immunohistochemical double staining of a skin tissue sample with green fluorescently labeled anti-MUC1/ZD-specific monoclonal antibody ZUM12D8 and red fluorescently labeled anti-MUC1 tandem-repeat-specific monoclonal antibody H23. A skin tissue sample was immunostained as described in Methods and in Figure 6 with a solution containing 40□g/ml of green fluorescently labeled (Molecular probes, AlexaFluor 488) anti-MUC1/ZD monoclonal antibody ZUM12D8 together with 10□g/ml of red fluorescently labeled (Molecular probes, AlexaFluor 546) anti-MUC1/REP tandem-repeat monoclonal antibody H23. The stained section was analyzed by confocal-laser microscopy calibrated for green fluorescence representing detection of MUC1/ZD (panels A and D), red fluorescence representing detection of MUC1/REP (B and E) and a merged overlay of the two signals (C and F). Panels A, B and C are 200 fold magnifications; panels C, D and E are 1000 fold magnifications.
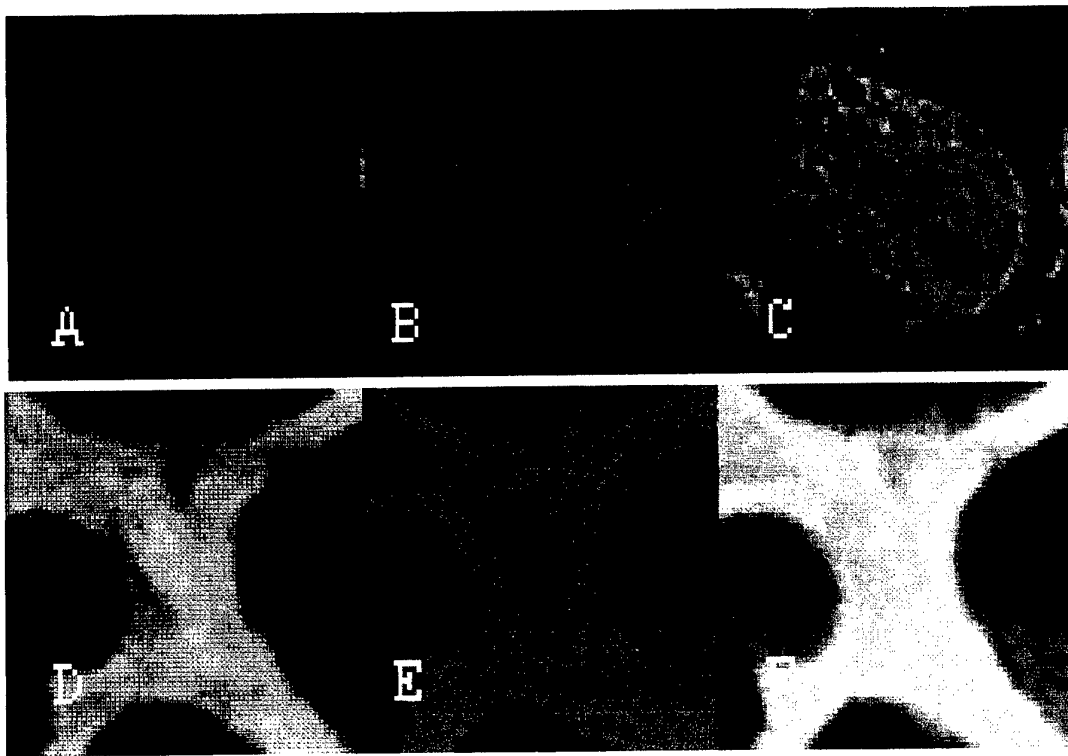
Figure 8. Homology of MUC1/ZD with proteins known to participate in cell defense. The query MUC1/ZD amino acid sequence was subjected to a blast search and the following hits appeared- **A.** CD14 protein with the following aliases: rat monocyte differentiation antigen CD14, LPS receptor, myeloid cell-specific leucine-rich glycoprotein (NCB™ accession no Q63691). **B.** A defensin-like peptide 1(Swiss-Prot™ accession no P82172). **C.** Plant defensin (NCB™ accession no CAA65045). **D.** Anaphylatoxin domain signature mouse CO4a (Swiss-Prot™ accession no P01029). The MUC1/ZD query sequence appears in the top row of each paired comparison, and the numbering for the MUC1/ZD protein here starts at the serine residue (S^{24} in figure 1B) immediately following cleavage of the MUC1 signal peptide- the isoleucine residue (I) initiating the MUC1/ZD specific sequence (that reads IPAPTTT...) is thus residue number 31. Identities are shown in red and conservative substitutions in blue. The most extensive similarity is seen between MUC1/ZD and CD14.

A.
```
Query:33          APTTTKSCR----ETFLKCFCRFINKGVFWASPILSSGQDLWWY
                  +P T + C+      E  ++C+C F +     W+S  L +G+D+ +Y
Sbjct:18          SPATPEPCELDQDEESVRCYCNFSDPQPNWSSAFLCAGEDVEFY
```

B.
```
Query:23 PSSTEK-NAIPAPTTTKSCRETFLK-CF 48
         P    E   N +       T +CRE FL   C+
Sbjct:  6 PRDCESINGVCRHKDTVNCREIFLADCY 33
```

C.
```
Query:40                    CRETFLKCFCR
                           CR   LKCFCR
Sbjct:61                    CRGFPLKCFCR
```

D.
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Query:40                    CRETFLKCFCR
                           CRE FL C C+
Sbjct:726                   CREPFLSC-CK
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Figure 9. Binding of bacteria to recombinant MUC1/ZD protein and to MUC1/ZD expressing 3T3 transfectants. (A) Recombinant MUC1/ZD was fluorescently labeled with AlexaFluor 488 as in Experimental Procedures and incubated with *Staphylococcus oxford* bacteria for 30' at 37°C. Binding of fluorescently labeled MUC1/ZD to the bacteria was analyzed by confocal microscopy (x100). Nomarski imaging (I, II). Overlay of the fluorescence and the Nomarski images (I', II'). **(B)** Mouse fibroblast NIH-3T3 cells were stably transfected with cDNA coding for MUC1/ZD. MUC1/ZD expression was determined with anti-MUC1/ZD monoclonal antibody ZUM12D8 by FACS analyses as in Methods. 3T3 cells (I). 3T3-MUC1/ZD transfectant cells (II). **Red line,** only secondary fluorescently labeled goat anti-mouse antibody; **Black line**, binding with mouse monoclonal antibody ZUM12D8 (anti-MUC1/ZD antibody). **(C)** Red fluorescently labeled *Pseudomonas aeruginosa* (as in Experimental Procedures) was added to 3T3-MUC1/ZD or control 3T3 cells. After 1h incubation at 25°C the cells were fixed with 2% paraformaldehyde and analyzed by confocal laser microscopy. Non-transfected 3T3 cells (I), 3T3-MUC1/ZD cells (II), addition of excess, competing unlabeled *Pseudomonas* aeruginosa together with labeled bacteria to 3T3-MUC1/ZD cells (III).
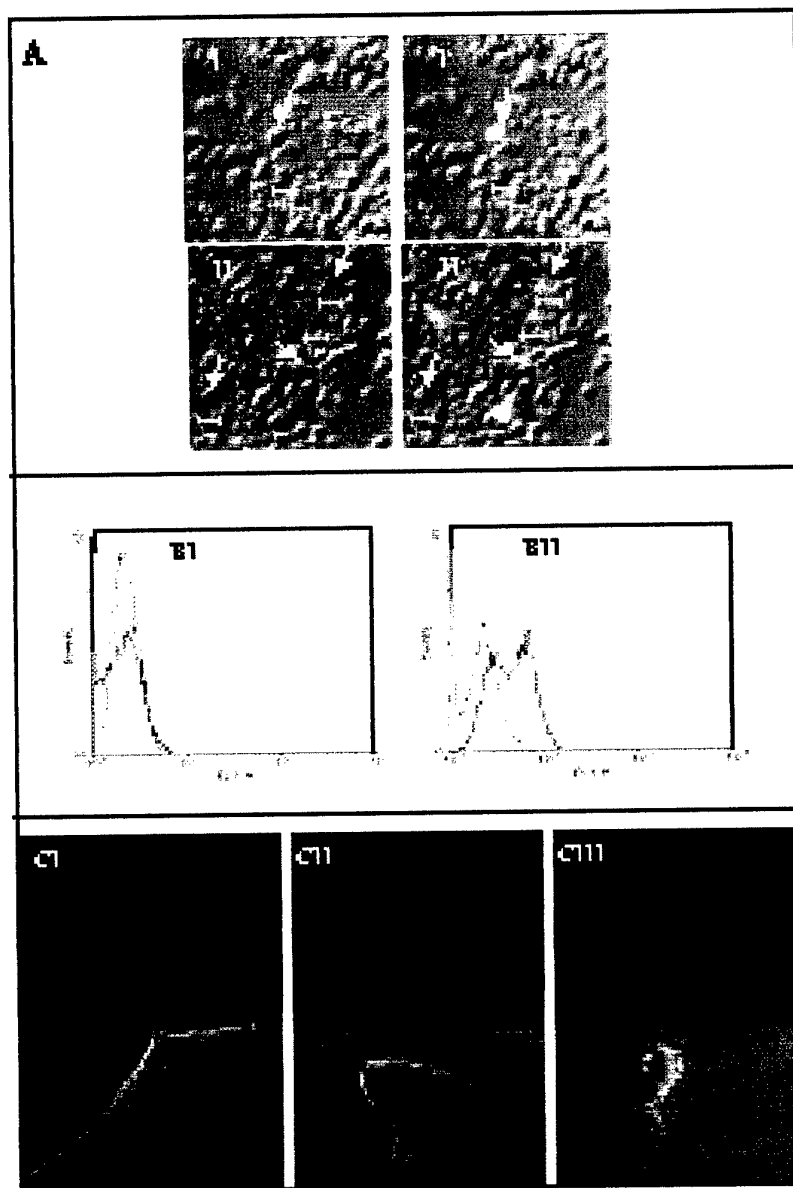
KEY RESEARCH ACCOMPLISHMENTS:

- Unequivocal demonstration of expression at the protein level of a novel MUC1 isoform, MUC1/ZD, in both cancer tissue and skin epithelial cells
- Generation of monoclonal antibodies specific for the MUC1/ZD
- Elucidation of function of novel MUC1/ZD isoform in binding to bacteria

REPORTABLE OUTCOMES

Levitin, F., Baruch, A., Weiss, M., Steigman, K., Hartman, M., Yoeli-Lerner, M. A., Ziv, R., Zrihan-Licht, S., Sheena, S., Gat, A., Lifschitz, B., Simha, M., Stadler, Y., Cholostoy, A., Gil, B., Greaves, D.R., Keydar, I., Smorodinsky, N., and **Wreschner, D.H.** *A novel protein isoform derived from the MUC1 gene by alternative splicing and frameshifting participates in bacterial recognition and binding.*

Submitted Manuscript, 2004

CONCLUSIONS

As described in our research proposal the *MUC1* gene is expressed in secretory epithelial tissues and at exceptionally high levels in human breast cancer cells. It is a well-known and widely accepted marker for breast cancer that can generate several functionally distinct MUC1 protein isoforms some containing a 20 amino acid tandem-repeat-array and additional forms lacking this domain. Findings deriving originally from our laboratory and subsequently confirmed by others, implicate the participation of the MUC1 proteins in signaling pathways. Analysis of MUC1 RT-PCR cDNA products revealed a supplementary and unique splice variant, MUC1/ZD, which is devoid of the tandem repeat array and utilizes a perfect out-of-frame splice acceptor site downstream to the tandem repeats. This splice event generates a secreted MUC1/ZD protein that harbors an N-terminal signal peptide and which contains a unique C-terminal stretch of 43 amino acids- this region shows marked homology with restricted regions of known cytokines and chemokines. We proposed to investigate the *hypothesis that the MUC1/ZD protein is a novel, biologically important, cytokine* by (a) analyzing MUC1/ZD protein expression in tumor tissues and correlating this expression with that of other MUC1 isoforms, (b) characterizing the signaling function of MUC1/ZD and proteins (receptor molecules?) with which the MUC1/ZD protein interacts, and (c) studying, both *in-vivo* and in *in-vitro*, the effect on tumor cell growth mediated by the MUC1/ZD protein.

Analysis of MUC1/ZD expression in tissues

To analyze MUC1/ZD expression we generated, purified and characterized monoclonal antibodies that specifically recognize the MUC1/ZD isoform. The two most promising monoclonal antibodies, designated ZUM12D8 and ZUM7E7, were chosen for a more detailed analysis. Both these monoclonal antibodies were found to react specifically by western blotting with recombinant, bacterial MUC1/ZD- they did not react with any other MUC1 isoform thus indicating that they should be useful reagents for investigating MUC1/ZD expression in tumors and other tissues. The monoclonal antibodies, both of which are of the IgGγ1 subtype, were purified utilizing Protein A Sepharose chromatography following which they were concentrated using a Centricon apparatus with a molecular weight cut-off of 5kDa. The ZUM12D8 monoclonal antibody retained good activity following this procedure, and was thus used to generate both biotin-labeled and fluorescently

labeled ZUM12D8 derivatives. Initial work carried out with the MUC1/ZD specific ZUM12D8 antibodies demonstrated that it could be used for immunohistochemical analyses, with the proviso that the sections used for staining were frozen sections and had been fixed with acetone and not with formaldehyde. As the *MUC1* gene is known to be expressed not only in breast tumor epithelial tissue but also in various other epithelial tissues (albeit at varying levels), we initially used the ZUM12D8 antibodies to stain frozen sections of skin as well as of tonsillar tissue (which contains pharyngeal epithelial cells). This analysis demonstrated that the MUC1/ZD protein is indeed expressed in both these tissues, and in both cases, is restricted to epithelial regions. Within the pharyngeal epithelium of the tonsil and the epidermis of the skin, MUC1/ZD expression is restricted to the basal cells (basal keratinocytes in the skin). Within the crypt epithelium, MUC1/ZD is expressed by the majority of epithelial cells. This finding was interesting from two different aspects:
  a) it indicated that MUC1/ZD expression is not restricted to breast epithelial cells, and
  b) that it likely plays a function in skin epithelial cells.

We then proceeded to perform much more extensive immunohistochemical analyses using green fluorescently labeled ZUM12D8. We concentrated our investigations on the expression of MUC1/ZD in skin epithelium as our preliminary investigations had shown that MUC1/ZD was expressed here at good levels. We also compared the expression levels of MUC1/ZD with expression of the classical MUC1 protein, the tandem repeat array membrane-bound MUC1/REP protein. To do this a MUC1/REP specific antibody called H23 was red fluorescently labeled. Using these two labeled antibodies (green for MUC1/ZD and red for MUC1/REP) we performed concurrent double-labelings of frozen skin tissue sections. These analyses demonstrated good expression of MUC1/ZD in the various layers of epidermal skin epithelial cells- MUC1/REP expression was not observed in these cells. MUC1/ZD expression was also significantly expressed in epithelial cells forming the sebaceous glands of the skin- interestingly MUC1/REP expression was also observed here but did not colocalize with MUC1/ZD expression. These analyses confirmed that MUC1/ZD is indeed expressed at the protein level in skin and furthermore indicated that MUC1/ZD expression and MUC1/REP expression do not necessarily go hand in hand. Closer inspection of the laser confocal analyses demonstrated that the MUC1/ZD protein localized very close to the cell membrane as well as in the extracellular space in close proximity to the cell membrane. As the MUC1/ZD protein comprises solely a signal peptide and does not have a transmembrane domain this was a surprising result and suggests that the MUC1/ZD protein may interact with extracellular surface components of the cell membrane.

In addition to the above work, we were also interested in analyzing the presence of the MUC1/ZD protein in body fluids and especially in secretions from breast tumor tissues. To accomplish this we established an ELISA assay that is specific for the MUC1/ZD protein. The ELISA assay developed is a sandwich assay in which a 96 well plate is coated with the MUC1/ZD specific monoclonal antibody ZUM7E7, followed by analyte application, and bound MUC1/ZD detected using biotinylated ZUM12D8, another monoclonal antibody which recognizes a different epitope on the MUC1/ZD protein. We have shown that this assay is specific for MUC1/ZD and shows a sensitivity down to 1ng/ml. We investigated sera from normal individuals as well as sera from breast cancer patients- we could not detect the MUC1/ZD protein. However we were able to clearly detect the MUC1/ZD protein in secretions obtained by drainage following a lumpectomy operation, thus confirming MUC1/ZD expression within the organism.

In our proposal we also wished to try to understand *the possible function of the MUC1/ZD molecule*. Clues to its function were derived by looking at the functions of other proteins harboring partial homologies with MUC1/ZD. This homology search showed limited similarities between the MUC1/ZD protein and proteins known to participate in cell defense against bacteria and fungi, such as the LPS receptor protein, CD14, a defensin-like peptide, a purothionine (plant defensin protein) and anaphylatoxin.

The common denominator for these proteins is that they all are secreted or membrane-associated proteins that participate in cell defense by functioning as antimicrobial proteins. Could it be that the MUC1/ZD secreted

protein is also participating in such activities?

This possibility becomes all the more intriguing when one considers that mucins have long been considered to perform a protective function by acting as shields at the apical surface of secretory epithelial cells. It is tempting to speculate that the *MUC1* gene generates, on the one hand, a classical membrane-bound mucin protein that protects the cell, and on the other hand, generates by alternative splicing a secreted protein, MUC1/ZD, that protects the cell by harboring anti-microbial activity. Using purified bacterially generated recombinant MUC1/ZD protein we were unable to demonstrate direct antimicrobial activity. In comparison CD14 also does not harbor direct antimicrobial activity but it does promote bacterial binding to the cell surface by recognizing components of the bacterial surface. We thus proceeded to investigate whether MUC1/ZD harbors a similar activity and promotes bacterial binding to cells that express MUC1/ZD.

We investigated this possibility by generating 3T3 fibroblast transfectants that express the MUC1/ZD protein. In correspondence to the immunohistochemical analyses, we found very little MUC1/ZD present in the cell culture medium of such transfectants. However, using flow cytometry analyses with the anti-MUC1/ZD monoclonal antibody ZUM12D8, the MUC1/ZD protein was readily detectable on the cell surface. We therefore investigated whether expression of the MUC1/ZD protein on the cell surface would support bacterial binding to these cells. Bacteria were fluorescently labeled and incubated with MUC1/ZD expressing 3T3 transfectants, the cells washed and then analyzed using confocal laser microscopy for bacterial binding. The MUC1/ZD transfectants readily bound the bacteria. No binding was observed with parental 3T3 cells that did not express the MUC1/ZD protein and addition of excess unlabelled bacteria competed out binding of the fluorescently labeled bacteria to the MUC1/ZD transfectants. These results demonstrate that expression of MUC1/ZD at, or in the proximity of, the cell surface can sustain bacterial binding to the cell surface.

In summary, we report a novel small protein derived from the *MUC1* gene by alternative splicing that does not contain the MUC1 tandem repeat array. This protein termed MUC1/Zs (MUC1/ZD) retains the same N-terminal MUC1 sequences as all MUC1 protein isoforms, that comprises the signal peptide and a subsequent stretch of thirty amino acids. The MUC1/ZD C-terminal 43 amino acids are novel and result from a reading frameshift engendered by a splicing event. Expression of MUC1/ZD was demonstrated by immunohistochemistry, immunoblotting, immunoprecipitation and an ELISA assay, using MUC1/ZD specific polyclonal and monoclonal antibodies. MUC1/ZD protein was expressed in cancerous tissues and epithelial cells comprising skin tissue- its expression did not parallel the mucinous MUC1 protein. MUC1/ZD protein is expressed in tissues as an oligomeric complex composed of disulfide-linked MUC1/ZD monomers. Limited homology between the novel MUC1/ZD C-terminal 43 amino acids and the N-terminal region of CD14, an innate immunity protein, prompted investigations as to whether MUC1/ZD binds to bacteria. Using transfectants expressing the MUC1/ZD protein we demonstrated that MUC1/ZD recognized and bound to bacterial cell surfaces. Results presented here demonstrate the existence of a novel MUC1 protein isoform, MUC1/ZD, expressed in breast cancer and skin epithelial cells that participates in bacterial recognition.